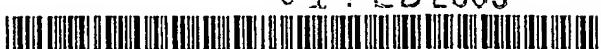


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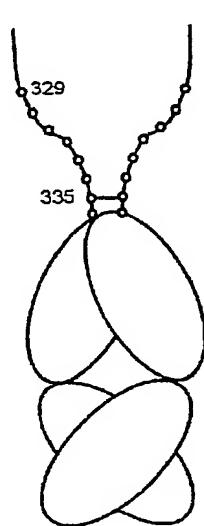
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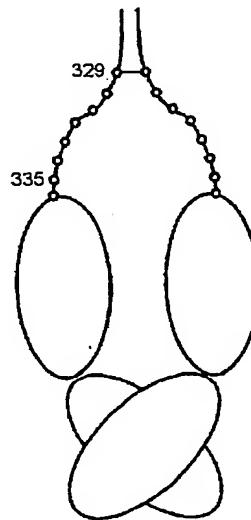
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Cys 335
Closed
Conformation

a



Cys 329
Open
Conformation

b

(57) Abstract: The present invention generally relates to mutant IgE proteins. Specifically, the present invention relates to modified IgE proteins that have reduced flexibility in their heavy chains in comparison to unmodified IgE proteins and are, as a result, constrained in a particular conformational state. The present invention also relates to three-dimensional models of IgE glycosylation mutants. The present invention also relates to the use of proteins of the instant invention to produce and isolate compounds that will inhibit the binding of IgE protein to Fc ϵ RI or Fc ϵ RI α . Also included, in the present invention are nucleic acid molecules encoding proteins of the instant invention. Also included are compounds which inhibit the binding of IgE to its receptor. The present invention also includes therapeutic compositions and kits comprising proteins and/or compounds of the instant invention as well as methods of treating an animal using such compositions and kits.

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MUTANTS OF IgE PROTEINS AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to mutant immunoglobulin epsilon (IgE) proteins
5 and three-dimensional models of such proteins. The invention also relates to the use of
the mutant proteins to identify compounds useful in the prevention and treatment of
allergy and the regulation of immune responses in an animal.

BACKGROUND OF THE INVENTION

Antibody Fc-receptors (FcRs) play an important role in the immune response by
10 coupling the specificity of secreted antibodies to a variety of cells of the immune system.
A number of cell types, including macrophages, mast cells, eosinophils, and basophils,
express membrane-bound FcRs on their surfaces. The binding of allergen-bound
antibodies to FcRs provides antigen-specificity to these cells, which upon activation
release further cell-specific mediators of the immune response, such as interleukins,
15 initiators of inflammation, leukotrienes, prostaglandins, histamines, or cytotoxic proteins.
The adoptive specificity of the FcRs allows a combinatorial approach to pathogen
elimination, by coupling the diversity of antibody antigen-recognition sites to the variety
of cell-types expressing these receptors.

FcR-initiated mechanisms are important in normal immunity to infectious disease
20 as well as in allergies, antibody-mediated tumor recognition, autoimmune diseases, and
other diseases in which immune responses are abnormal (i.e., not properly regulated).
Recent experiments with transgenic mice have demonstrated that the FcRs control key
steps in the immune response, including antibody-directed cellular cytotoxicity and
inflammatory cascades associated with the formation of immune complexes; see, for
25 example, Ravetch et al., 1998, *Annu Rev Immunol* 16, 421-432. Receptors that bind IgG
(Fc_gRI, Fc_gRII, and Fc_gRIII, known collectively as Fc_gRs) mediate a variety of
inflammatory reactions, regulate B-cell activation, and also trigger hypersensitivity
reactions. The high affinity Fc epsilon receptor (also known as the IgE receptor or Fc_eRI)
is associated with the activation of mast cells and the triggering of allergic reactions and
30 anaphylactic shock. Knockout mice for the Fc_eRI alpha chain (Fc_eRI α) are unable to
mount IgE-mediated anaphylaxis (see for example, Dombrowicz et al., 1993, *Cell* 75,
969-976), although Fc_gRs are still able to activate mast cells (see, for example,
Dombrowicz et al., 1997, *J. Clin. Invest.* 99, 915-925; Oettgen et al., 1994, *Nature* 370,

367-370). Fc ϵ RI has also been shown to trigger anti-parasitic reactions from platelets and eosinophils as well as deliver antigen into the MHC class II presentation pathway for the activation of T cells; see, for example, Gounni et al., 1994, *Nature* 367, 183-186; Joseph et al., 1997, *Eur. J. Immunol.* 27, 2212-2218; Maurer et al., 1998, *J. Immunol.* 161, 2731-2739. The beta subunit of Fc ϵ RI has been associated with asthma in genetic studies; see, for example, Hill et al., 1996, *Hum. Mol. Genet.* 5, 959-962; Hill et al., 1995, *Bmj* 311, 776-779; Kim et al., 1998, *Curr. Opin. Pulm. Med.* 4, 46-48; Mao et al., 1998, *Clin. Genet.* 53, 54-56; Shirakawa et al., 1994, *Nat. Genet.* 7, 125-129. A significant fraction of the population (~20%) may be affected by allergies, and this century has seen a substantial increase in asthma. Since IgE binding to Fc ϵ RI is a requisite event in the reaction to different allergens, therapeutic strategies aimed at inhibiting Fc ϵ RI/IgE interactions could provide a useful treatment for these diseases. For example, monoclonal antibodies that target IgE and block receptor binding have shown therapeutic potential; see, for example, Heusser et al., 1997, *Curr. Opin. Immunol.* 9, 805-813.

Fc ϵ RI is found as a tetrameric (abg₂) or trimeric (ag₂) membrane bound receptor on the surface of mast cells, basophils, eosinophils, langerhans cells and platelets. The alpha chain, also referred to as Fc ϵ RI α , of Fc ϵ RI binds IgE molecules with high affinity (K_A of about 10^9 to 10^{10} moles/liter (M)), and can be secreted as a 172-amino acid soluble, IgE-binding fragment by the introduction of a stop codon before the single C-terminal transmembrane anchor; see, for example, Blank et al., 1991, *E. J. Biol. Chem.* 266, 2639-2646, which describes the secretion of a soluble IgE-binding fragment of 172 amino acids. The extracellular domains of the human Fc ϵ RI α protein belong to the immunoglobulin (Ig) superfamily and contain seven N-linked glycosylation sites. Glycosylation of Fc ϵ RI α affects the secretion and stability of the receptor, but is not required for IgE-binding; see, for example, LaCroix et al., 1993, *Mol. Immunol.* 30, 321-330; Letourneur et al., 1995, *J. Biol. Chem.* 270, 8249-8256; Robertson, 1993, *J. Biol. Chem.* 268, 12736-12743; Scarselli et al., 1993, *FEBS Lett* 329, 223-226. The beta and gamma chains of Fc ϵ RI are signal transduction modules.

Prior investigators have disclosed the nucleic acid sequence encoding the proteins for human Fc ϵ RI α ; see, for example, U.S. Patent No. 4,962,035, by Leder, issued October 9, 1990; U.S. Patent No. 5,639,660, by Kinet et al., issued June 17, 1997; Kochan et al., 1988, *Nucleic Acids Res.* 16, 3584; Shimizu et al., 1988, *Proc. Natl. Acad. Sci. USA* 85, 1907-1911; and Pang et al., 1993, *J. Immunol.* 151, 6166-6174. Nucleic acid sequences

have also been reported for nucleic acid molecules encoding the human Fc ϵ RI beta and gamma chains; see, respectively, Kuster et al., 1992, *J. Biol. Chem.* 267, 12782-12787; Kuster et al., 1990, *J. Biol. Chem.* 265, 6448-6452. Nucleic acid sequences have also been reported for nucleic acid molecules encoding canine Fc ϵ RI α , murine Fc ϵ RI α , rat 5 Fc ϵ RI α , feline Fc ϵ RI α and equine Fc ϵ RI α proteins; see, respectively, GenBank™ accession number D16413; Swiss-Prot accession number P20489 (represents encoded protein sequence); GenBank accession number J03606; PCT Publication No. WO 98/27208, by Frank et al., published June 25, 1998, referred to herein as WO 98/27208; and PCT Publication No. WO 99/38974, by Weber et al., published August 5, 1999, 10 referred to herein as WO 99/38974. In addition, methods to detect IgE antibodies using a Fc ϵ RI α protein have been reported in PCT Publication No. WO 98/23964, by Frank et al., published June 4, 1998, referred to herein as WO 98/23964; WO 98/27208, *ibid.*; PCT Publication No. WO 98/45707, by Frank et al., published October 15, 1998, referred to herein as WO 98/45707; and WO 99/38974, *ibid.*. WO 98/23964, WO 98/27208, WO 15 98/45707 and WO 99/38974 are each incorporated by reference herein in its entirety.

IgE shares the general overall structure common to all immunoglobulin molecules in that it is composed of four polypeptide chains: two identical heavy chains (H chains) and two identical light chains (L chains) (reviewed in Immunobiology: The Immune System in Health and Disease, Janeway and Travers, 1996, Garland Publishing Inc. New York which is incorporated by reference in its entirety). The two heavy chains, which have a molecular weight of approximately 65 kDa each in their unglycosylated form, are linked by disulfide bonds. Each heavy chain is further linked to a light chain, each light chain having a molecular weight of approximately 25 kDa, resulting in the final, four chain molecule. Both the heavy and light chains contain distinct sequence domains. The 20 light chains contain two domains, a variable domain (referred to as V_L), with the sequence in this region varying between different antibodies of the same isotype, and a constant domain (referred to as C_L), with the sequence in this region remaining constant between different antibodies of the same isotype. The number of sequence domains in the heavy 25 chains varies between the different isotypes. The heavy chain polypeptide of IgE has five sequence domains consisting of one variable sequence domain (referred to as V_H), and four constant sequence domains (referred to as C_{H1}-C_{H4}). It is the V_L and V_H domains which are involved in antigen recognition whereas the constant domains of the heavy 30 chains impart the distinctive functional properties (e.g., receptor binding) of an

immunoglobulin class to the molecule and determine to which of the five main immunoglobulin classes the molecule belongs.

In addition to the heavy and light protein components, all immunoglobulins also contain significant amounts of carbohydrate in the form of simple and complex side chains covalently linked to amino acids in the polypeptide chains. The carbohydrate side chains are usually attached via an N-glycosidic linkage, although O-glycosidic linkages have been observed. In general, carbohydrate side chains are attached to the protein portion of the molecule via linkages located in one of the constant domains of the heavy chain although exceptions to this rule have been seen. The number of glycosidic linkages varies between and within immunoglobulin types, although in general, IgE is believed to have an average of 5 oligosaccharides per complete immunoglobulin molecule

IgE antibodies interact with the cellular receptors Fc ϵ RI α and Fc ϵ RII (CD23) through amino acid sequences present in the constant domains of the heavy chains. There have been several reports of the use of mutagenesis and swapping techniques to attempt to identify amino acids of either Fc ϵ RI α or IgE involved in the binding of (i.e., interaction between) those respective proteins, reports attempting to model Fc ϵ RI α proteins based on homology to other Ig-superfamily members, and reports that identify compounds that apparently inhibit such binding; see, for example, Cook et al., 1997, *Biochemistry* 36, 15579-15588; Hulett et al., 1994, *J. Biol. Chem.* 269, 15287-15293; Hulett et al., 1995, *J. Biol. Chem.* 270, 21188-21194; Mallamaci et al., 1993, *J. Biol. Chem.* 268, 22076-22083; Robertson, 1993, *ibid.*; Scarselli et al., 1993, *ibid.* McDonnell et al., 1997, *Biochem. Soc. Trans.* 25, 387-392; McDonnell et al., 1996, *Nat. Struc. Biol.* 3, 419-426; PCT Publication No. WO 97/40033, by Cheng et al., published October 30, 1997; U.S. Patent No. 5,180,805, by Gould et al., issued January 19, 1993; U.S. Patent No. 5,693,758, by Gould et al., issued December 2, 1997; PCT Publication No. WO 96/01643, by Gould et al., published January 25, 1996; PCT Publication No. WO 95/14779, by Gould et al., published June 1, 1995. Recent crystallographic examination of the individual molecules as well as the IgE/Fc ϵ RI α complex has elucidated the specific amino acids involved in the interaction between IgE and Fc ϵ RI α ; see, for example, PCT Publication No. WO 00/26246, Jardetzky et al., published May 11, 2000; U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published January 2, 2003; PCT Publication No. WO 01/69253 A3, Jardetzky et al., published September 20, 2001; U.S. Patent Publication No.

20010039479, Jardetzky et al., published November 8, 2001; and PCT Publication No. WO 01/68861 A3, Jardetzky et al., published September 20, 2001, each of which is incorporated herein by reference in its entirety. The three-dimensional models generated by these studies have shown that the binding of Fc ϵ RI α to IgE is mediated by interactions of amino acids in Fc ϵ RI α with amino acids in the C ϵ 2/C ϵ 3 linker region (also referred to as the flexible linker region) and in the C ϵ 3 domains of the IgE heavy chain. Furthermore, the 3-dimensional models of IgE demonstrate that the heavy chains of IgE are flexible and can adopt at least two unique conformations, a closed conformation and an open conformation. The two conformations can be distinguished, in part, by the relative spatial orientation of the C ϵ 3 domains which are further apart in the open conformation and closer together in the closed conformation. In the closed conformation, the C ϵ 3-C ϵ 4 interdomain angle is more acute than that observed between homologous IgG-Fc domains (Deisenhofer et al., 1976; Harris et al., 1999) or in the Fc ϵ RI-bound IgE-Fc (open conformation, Garman et al., 2000). Additionally, both the relative disposition of the two C ϵ 3 domains with respect to each other and to the C ϵ 4 domains is altered. In the closed structure, the IgE-Fc C ϵ 3 domains are closer together and slightly rotated with respect to each other. In addition, the distance between the first residue (the N-terminal amino acid) of the C ϵ 3 A strands is only about 13 Å to about 22 Å. The distance increases to 23 Å in the open conformation of IgE-Fc, which is similar to the 22 Å observed between the Cy domains in the IgG2a-Fc (Harris et al., 1997). In the change between the open and closed conformations, the top of each C ϵ 3 domain moves about 10 Å towards the other C ϵ 3 domain across the dimer axis and 8 Å towards the C ϵ 4 domain of the same chain.

Studies of the IgE/Fc ϵ RI α complex have revealed the IgE-Fc molecule is in the open conformation when bound to Fc ϵ RI α . The large conformational change of the IgE-Fc structure reorients loops in the C ϵ 3 domain that interact with the high affinity receptor, Fc ϵ RI. The large movement of the Fc ϵ RI-binding loops suggests that they would be poorly positioned in the closed IgE-Fc structure to interact with the receptor. In the open form, the receptor-binding loops are exposed and the binding residues display a large concave surface that is available to interact with Fc ϵ RI α . Based in part on these data, it is predicted that if IgE molecules are forced to adopt the closed configuration, due to binding of a compound for example, these closed IgE molecules would be unable to bind to Fc ϵ RI α . Further, using these data, it is possible to design tools (e.g. mutant proteins)

useful for the production of compounds that bind IgE, locking it in the closed conformation thereby preventing it from binding to its receptor.

While studies to date have provided information on the structure of IgE and Fc ϵ RI α as well as the nature of their interaction, the need remains for specific tools useful 5 for the discovery and production of compounds with which to treat and diagnose allergy and to regulate immune responses. Also needed are safe and efficacious compounds to prevent or treat allergy and to regulate other immune responses in an animal.

SUMMARY OF THE INVENTION

The present invention generally relates to mutant, IgE heavy chain proteins, IgE 10 proteins comprising mutant IgE_{HC} proteins and 3-dimensional models of such mutant IgE proteins. Specifically, the present invention relates to mutant IgE proteins that have reduced flexibility in their heavy chains in comparison to the native molecule and are, as a result, constrained in a particular conformational state. The present invention also relates to 3-dimensional models of IgE glycosylation mutants. IgE and IgE_{HC} mutants 15 can be used, for example, to isolate or produce compounds that regulate the IgE-mediated immune response in an animal. The present invention also relates to the use of mutant IgE_{HC} and mutant IgE proteins of the instant invention to produce and isolate compounds that will inhibit the binding of IgE to Fc ϵ RI or Fc ϵ RI α . Included in the present invention are nucleic acid molecules encoding proteins of the instant invention as well as cells and 20 recombinant viruses comprising such nucleic acid molecules. Also included are compounds which inhibit the binding of IgE to Fc ϵ RI or Fc ϵ RI α . The present invention also includes therapeutic compositions and kits comprising proteins and/or compounds of the instant invention as well as methods of treating an animal using such compositions and kits. Accordingly, the present invention builds on the teaching of PCT Publication 25 No. WO 00/26246, Jardetzky et al., published May 11, 2000; U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published January 2, 2003; PCT Publication No. WO 01/69253 A3, Jardetzky et al., published September 20, 2001; U.S. Patent Publication No. 20010039479, Jardetzky et al., published November 8, 2001; and PCT Publication No. WO 01/68861 A3, Jardetzky et al., published September 20, 2001.

30 **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows a comparison of the inter-chain and inter-residue distance in the open and closed conformations of IgE-Fc. Fig. 1a shows a side view of the Ce3 and Ce4 domains in the closed conformation. Fig. 1b shows a top view of the Ce3 domains in the

closed conformation. The solid line indicates the 13 Å gap between the N-terminal amino acids (residues 336) of each chain. Fig. 1b shows a side view of the Ce3 and Ce4 domains in the open conformation. Fig. 1d shows a top view of the Ce3 domains in the open conformation. The solid line indicates the 23.5 Å gap between the N-terminal 5 amino acids (residues 336) of each chain.

Fig. 2 shows a schematic representation of the open and closed IgE conformations, highlighting the potential disulfide bonds that could be formed by the cysteine residues created in the IgE_{HC} mutants (e.g. disulfide bonds at amino acid positions 329 and 335 are shown). Fig. 2a shows the Ce4 domain and residues 329-336 of 10 the Ce3 domain in the closed conformation. The solid line represents a disulfide bond between residues 335 of each heavy chain. Fig. 2b shows the Ce4 domain and residues 329-336 of the Ce3 domain in the open conformation. The solid line represents a disulfide bond between residues 329 of each heavy chain.

Fig. 3 shows a western blot of the IgE_{HC} cysteine mutant proteins separated by 15 PAGE under reducing and non-reducing conditions. Numbers above each lane indicate the amino acid residue in IgE_{HC} changed to a cysteine with the potential to form an interchain disulfide bond. The position of protein monomers and dimers are indicated by the arrows on the left side of the figure. Fig. 3a shows the proteins exist in both monomeric and dimeric forms when analyzed under non-reducing conditions. Fig. 3b 20 shows that when the IgE-Fc proteins are subjected to reducing conditions, they all exist in the monomeric form.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to mutant IgE proteins, mutant IgE_{HC} 25 proteins and 3-dimensional models of such mutant IgE proteins. In describing the present invention, certain terms used herein are defined as follows:

As used herein, the term "a" or "an" entity refers to one or more of that entity; for example, a protein refers to one or more proteins or at least one protein; as another example, a nucleic acid molecule refers to one or more nucleic acid molecules or at least one nucleic acid molecule. As such, the terms "a" or "an", "one or more", and "at least 30 one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably herein.

As used herein, the terms "IgE heavy chain", "IgE_{HC}" and "IgE_{HC} protein" can be used interchangeably and refer to a protein from any animal that comprises an IgE Fc

receptor binding site and, as such, contains at least a portion of Ce3, with or without at least a portion of Ce4. An IgE_{HC} protein can be full-length immunoglobulin epsilon (IgE) heavy chain, a full-length Fc region of an IgE heavy chain, a fragment of an IgE Fc region that binds to Fc_εRI or Fc_εRIα, or any protein comprising a fragment of an IgE Fc region that binds to Fc_εRI or Fc_εRIα.

As used herein, the terms "IgECe3", "Ce3" and "Ce3 domain" can be used interchangeably and refer to the third constant region domain of the full length IgE heavy chain protein.

As used herein, the term "IgECe4", "Ce4" and "Ce4 domain" can be used 10 interchangeably and refer to the fourth constant region domain of the full length IgE heavy chain protein.

As used herein, the term "IgECe3/Ce4" refers to a protein comprising at least a portion of IgECe3 and at least a portion of IgECe4.

As used herein, the term "Ce2/Ce3 linker region" refers to the amino acid segment 15 between the second and third constant domains.

As used herein, the term "IgE Fc region" refers to the region of the IgE heavy chain consisting of the second, third and fourth constant domains, Ce2, Ce3 and Ce4.

As used herein, the term "IgE protein" refers to a molecule comprising at least one or more IgE_{HC} proteins, either alone or in combination with one or more IgE light chains. 20 One example of an IgE protein is an IgE antibody comprising two heavy chains and two light chains, with another example being an IgE_{HC} dimer.

As used herein, the term "unmodified", as applied to IgE proteins, refers to IgE proteins having Fc_εRI or Fc_εRIα binding activity identical to the Fc_εRI or Fc_εRIα binding activity of an IgE protein comprising an IgE_{HC} that comprises the amino acid sequence of SEQ ID NO:11. As applied to IgE_{HC}'s and related nucleic acid molecules (i.e. nucleic acid molecules encoding such IgE_{HC}'s), the term "unmodified" refers to IgE_{HC}'s and related nucleic acid molecules having functional characteristics identical to those possessed by an IgE_{HC} or a related nucleic acid molecule comprising amino acid sequence SEQ ID NO:11 or nucleotide sequence SEQ ID NO:10, respectively.

As used herein, the term "mutant" refers to IgE proteins, IgE_{HC} proteins and 30 related nucleic acid molecules having sequences similar to unmodified IgE proteins, IgE_{HC} proteins and related nucleic acid molecules but that differ functionally from their unmodified counterparts. Specifically, mutant IgE proteins, mutant IgE_{HC}'s and related

nucleic acid molecules (i.e. nucleic acid molecules encoding such IgE_{HC}'s) do not bind Fc ϵ RI in the same manner as an unmodified counterpart. Mutants may be isolated from nature or may be created as a result of manipulation.

As used herein, the term "isolated" refers to IgE proteins, IgE_{HC} proteins and related nucleic acid molecules that have been removed from their natural milieu. As such, the term "isolated" does not necessarily reflect the extent to which the IgE proteins, IgE_{HC} proteins and related nucleic acid molecules have been purified. An isolated IgE protein, IgE_{HC} protein or related nucleic acid molecules can be obtained from its natural source or it can be produced using recombinant technology and/or through the use of chemical synthesis or modification.

As used herein, the term "closed conformation" refers to the 3-dimensional conformation of an IgE protein in which the constant domains of the IgE_{HC} proteins are oriented in such a way as to prevent binding of the IgE protein to an Fc ϵ RI or Fc ϵ RI α . IgE proteins in a closed conformation have IgE_{HC} proteins in which the N-terminal amino acid of the C ϵ 3 domains are less than 23 angstroms (\AA) apart.

As used herein, the term "open conformation" refers to a 3-dimensional conformation of an IgE protein in which the constant domains of the IgE_{HC} proteins are oriented in such a way as to allow binding of the IgE molecule to a Fc ϵ RI or Fc ϵ RI α . An IgE protein in the open conformation is one in which the N-terminal amino acid of the C ϵ 3 domains are at least 23 \AA apart.

As used herein, the term "spatial mobility" refers to the ability of the IgE_{HC} proteins to move or shift their position relative to one another and/or the rest of the IgE protein.

As used herein, the term "reduced spatial mobility" refers to an IgE protein that is in one conformation, either open or closed, and in which the motion of the IgE_{HC}'s are restricted so that the IgE protein is unable to adopt the alternative conformation. For example, an IgE in the closed conformation having reduced spatial mobility is unable to alter its conformation so that the N-terminal amino acids of the C ϵ 3 domains are at least 23 \AA apart. Similarly, the term "constrained" refers to an IgE that is in one conformation, either open or closed, and is unable to adopt the alternative conformation.

As used herein, the term "IgE open form mutants" (IgE_{ofm}) refers to mutant IgE proteins that are constrained in the open conformation and have reduced spatial mobility such that they are unable to adopt the closed conformation.

As used herein, the term "IgE closed form mutants" (IgE_{cfm}) refers to mutant IgE proteins that are constrained in the closed conformation and have reduced spatial mobility such that they are unable to adopt the open conformation.

As used herein, the term "IgE glycosylation mutant" (IgE_{gm}) refers to mutant IgE proteins in which the amino acid sequence of one or more N-linked glycosylation sites has been altered so that the site is no longer an N-linked glycosylation site (i.e. the mutated site can no longer be glycosylated by the N-linked glycosylation method).

As used herein, the term "extracellular domain of a Fc ϵ RI α protein" is the portion of the Fc ϵ RI alpha chain that is exposed to the environment outside the cell and that binds to the Fc domain of an IgE antibody. Such an Fc ϵ RI α extracellular domain can be (a) a complete extracellular domain which is a domain that extends from the first amino acid of a mature Fc ϵ RI alpha chain through the last amino acid prior to the start of the transmembrane region or an extracellular domain that is functionally equivalent, in that such a domain includes D1 and D2 domains, and displays a similar affinity for the IgE antibody to which such an Fc ϵ RI α protein naturally binds or (b) a fragment of any of the extracellular domains of (a), wherein the fragment retains its ability to bind to the Fc domain of an IgE antibody.

In one aspect, the present invention provides isolated mutant IgE proteins comprising IgE_{HC}'s having reduced spatial mobility in comparison to the spatial mobility of the IgE_{HC}'s in an unmodified IgE protein. Suitable mutant IgE proteins are those in which the spatial mobilities of the IgE_{HC} Ce3 and Ce4 domains within the IgE protein have been restricted due to modification of the IgE_{HC}'s. Previous crystallographic analysis of the unmodified IgE protein Ce3 and Ce4 domains has demonstrated these domains can move within the IgE protein causing the IgE protein to adopt one of at least two conformations: an open conformation or a closed conformation. In the closed conformation, the N-terminal amino acid residues of the Ce3 domains of the two heavy chains, which correspond to the amino acid in position 9 of SEQ ID NO:11, which itself corresponds to position 336 of the full-length human IgE heavy chain protein, have an inter-residue distance of about 13 Å whereas in the open conformation, the two Ce3 domain N-terminal amino acid residues are positioned so that they have an inter-residue distance of at least 23 Å. The IgE protein is in the open conformation when bound to Fc ϵ RI α and, based on the structural data, it is predicted that IgE proteins in the closed conformation would be unable to bind an Fc ϵ RI or Fc ϵ RI α .

One embodiment of the present invention is a mutant IgE protein comprising an IgE_{HC} that has been modified in such a way as to cause the IgE protein to be constrained in the closed conformation. IgE proteins constrained in the closed conformation have IgE_{HC}'s in which the N-terminal amino acid residues of the Cε3 domains are less than 23Å apart; such mutant IgE proteins are unable to flex significantly enough to allow an inter-residue distance of 23Å or more. Suitable IgE proteins of the present invention include those in which the N-terminal amino acid residues of the IgE_{HC} Cε3 domains can achieve an inter-residue distance of from about 13Å to less than 23Å. As such, useful mutant IgE proteins of the present invention include those in which the N-terminal amino acid residues of the IgE_{HC} Cε3 domains can achieve an inter-residue distance of no greater than about 13Å, no greater than about 14Å, no greater than about 15Å, no greater than about 16Å, no greater than about 17Å, no greater than about 18Å, no greater than about 19Å, no greater than about 20Å, no greater than about 21Å or no greater than about 22Å or less than 23Å. Such mutant IgE proteins can be isolated from natural sources or they can be produced by laboratory manipulation of unmodified IgE proteins, IgE_{HC}'s and/or related nucleic acid molecules. Suitable methods to modify an IgE protein are known to those skilled in the art and include, but are not limited to, chemical or enzymatic modification of the IgE_{HC}'s, alteration of the IgE_{HC} protein sequences to produce a mutant protein giving the desired conformation, alteration of a nucleic acid molecule encoding IgE_{HC}'s and combinations thereof; examples of such methods are described in more detail herein. A particularly useful mutant IgE protein is one that is constrained in the closed conformation and is unable to adopt the open conformation and as a result, is unable to bind to a FcεRI or FcεRIa.

One embodiment of the present invention is a mutant IgE protein comprising an IgE_{HC} that comprises an amino acid sequence having at least 50% identity to SEQ ID NO:11, wherein the amino acid sequence of the mutant IgE_{HC} has been modified to allow the IgE_{HC}'s to form cross-linking covalent bonds thereby constraining movement of such IgE_{HC}'s in an IgE protein. A suitable type of bond to form is a disulfide bond. Methods to determine percent identities and similarities are well known to those skilled in the art. Examples of mutant IgE proteins include, but are not limited to, those comprising an IgE_{HC} that comprises an amino acid sequence at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98% or at least about 100% identical to

SEQ ID NO:11, but wherein the amino acid residue in the IgE_{HC} corresponding to position 2, 3, 4, 5, 6, 7, 8, or 9 of SEQ ID NO:11 is a cysteine or a methionine.

In one embodiment, a mutant IgE protein of the present invention comprises an IgE_{HC} that comprises an amino acid sequence at least about 80%, at least about 85%, at 5 least about 90%, at least about 95%, at least about 98% or at least about 100% identical to an amino acid sequence selected from the group consisting of:

- (a) SEQ ID NO:13, wherein the amino acid residue in said IgE_{HC} corresponding to position 2 of SEQ ID NO:13 is a cysteine or a methionine;
- (b) SEQ ID NO:15, wherein the amino acid residue in said IgE_{HC} corresponding to 10 position 3 of SEQ ID NO:15 is a cysteine or a methionine;
- (c) SEQ ID NO:17, wherein the amino acid residue in said IgE_{HC} corresponding to position 4 of SEQ ID NO:17 is a cysteine or a methionine;
- (d) SEQ ID NO:19, wherein the amino acid residue in said IgE_{HC} corresponding to position 5 of SEQ ID NO:19 is a cysteine or a methionine;
- 15 (e) SEQ ID NO:21, wherein the amino acid residue in said IgE_{HC} corresponding to position 6 of SEQ ID NO:21 is a cysteine or a methionine;
- (f) SEQ ID NO:23, wherein the amino acid residue in said IgE_{HC} corresponding to position 7 of SEQ ID NO:23 is a cysteine or a methionine;
- (g) SEQ ID NO:25, wherein the amino acid residue in said IgE_{HC} corresponding to 20 position 8 of SEQ ID NO:25 is a cysteine or a methionine; and
- (h) SEQ ID NO:27, wherein the amino acid residue in said IgE_{HC} corresponding to position 9 of SEQ ID NO:27 is a cysteine or a methionine.

In a particularly useful embodiment, a mutant IgE protein comprises an IgE_{HC} that comprises an amino acid sequence selected from the group consisting of SEQ ID NO:13, 25 SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:27.

The present invention also provides an isolated mutant IgE_{HC} modified in such a way as to cause an IgE protein comprising the mutant IgE_{HC} to have reduced spatial mobility in comparison to the spatial mobility of an IgE protein comprising an 30 unmodified IgE_{HC}. A suitable mutant IgE_{HC} is one which causes an IgE protein to be constrained in an open or closed conformation, with the closed conformation being preferred. Suitable mutant IgE_{HC}'s of the present invention comprise an amino acid sequence having at least 50% identity to SEQ ID NO:11, but wherein the IgE_{HC} has been

modified, allowing the formation of covalent bonds between two or more IgE_{HC}'s thereby constraining the movement of such IgE_{HC}'s in an IgE protein. Preferred mutant IgE_{HC}'s, while they no longer bind Fc ϵ RI or Fc ϵ RI α with the affinity of an IgE_{HC} comprising SEQ ID NO:11, do retain other functions of IgE_{HC}'s, for example the ability to elicit an immune response to an unmodified IgE_{HC} and the ability to bind to antibodies generated using an unmodified IgE_{HC}. Any unmodified or mutant IgE protein or IgE_{HC} or related nucleic acid molecule can be subjected to modification to produce a mutant IgE or a mutant IgE_{HC} or related nucleic acid molecule of the present invention. Preferred proteins or nucleic acid molecules to modify are mammalian, with human, non-human primate, feline, canine, equine, murine, ovine, bovine or porcine protein or nucleic acid molecules being preferred. Particularly preferred are human, feline, canine or equine proteins or nucleic acid molecules with human proteins or nucleic acid molecules being more preferred. Methods of modifying IgE_{HC}'s to allow the formation of inter-chain covalent bonds, such as, but not limited to disulfide bonds, are known to those skilled in the art and include, but are not limited to, chemical and/or enzymatic modification of the IgE_{HC}'s, alteration of the IgE_{HC} protein sequence through the use of protein or recombinant technologies, and combinations thereof. A suitable type of bond to form is a disulfide bond. Any method of modification that causes an IgE protein comprising the modified IgE_{HC} to be constrained in the closed conformation may be used to produce IgE_{HC}'s and IgE proteins of the present invention. A particularly useful embodiment is one that modifies the IgE_{HC} in such a way as to result in the N-terminal amino acids of the C ϵ 3 domains in an IgE protein comprising the modified IgE_{HC} being no more than about 13 \AA , no more than about 14 \AA , no more than about 15 \AA , no more than about 16 \AA , no more than about 17 \AA , no more than about 18 \AA , no more than about 19 \AA , no more than about 20 \AA , no more than about 21 \AA , no more than about 22 \AA apart, or less than 23 \AA apart.

One example of a useful method of modification is a chemical or enzymatic treatment. Suitable chemical or enzymatic treatments are well known to those skilled in the art and include, but are not limited to, for example, glycosylation reactions, myristilation reactions, biotinylation reactions, reduction reactions, oxidation reactions, protease reactions and the like. A suitable treatment is one which cross-links the IgE_{HC}'s thereby constraining an IgE protein comprising the modified IgE_{HC} in the closed conformation.

In one embodiment, an IgE_{HC} is modified by altering the amino acid sequence. A mutant IgE_{HC} may have one or more sequence differences compared to the unmodified IgE_{HC} sequence and these sequence differences may arise naturally or they may be introduced through laboratory manipulation (e.g. substitutions, insertions, deletions) of an IgE_{HC} protein or a nucleic acid molecule encoding such an IgE_{HC}. Any alteration in the IgE_{HC} amino acid sequence that results in an IgE protein that comprises the mutant IgE_{HC} being constrained to the closed conformation are suitable. Such sequence alterations can be made at any location in the IgE_{HC} that results in the IgE protein being constrained in the closed conformation. Particularly suitable positions at which to make modifications are positions in the Ce2/Ce3 linker region, the Ce3 domain and/or the Ce4 domain. For example, according to U.S. Patent Publication No. 20010039479-A1, Jardetzky et al., published on November 8, 2001, and U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published on January 2, 2003, the human IgE_{HC} Ce3 domain spans amino acids 9 through 107 of SEQ ID NO:11, which correspond to amino acids 336 through 434 of full length human IgE_{HC}, using the numbering system of Dorrington et al., 1978, *Immunol Rev* 41, 3-25, which is incorporated herein by reference in its entirety, while the Ce4 domain spans amino acids 114 through 220 of SEQ ID NO:11, which correspond to amino acids 441 through 547 of full length human IgE_{HC}, using the numbering system of Dorrington et al., 1978, *Immunol Rev* 41, 3-25. As such, IgE Ce3/Ce4 extends from amino acids 9-220 of SEQ ID NO:11. A suitable embodiment is an IgE_{HC} in which the amino acid sequence of the Ce2/Ce3 linker region is altered by the addition, substitution or deletion of amino acids. For example, according to U.S. Patent Publication No. 20010039479-A1, Jardetzky et al., published on November 8, 2001, and U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published on January 2, 2003, the IgE_{HC} Ce2/Ce3 linker region spans amino acids 1-8 of SEQ ID NO:11, which correspond to amino acids 328 through 335 of the full length human IgE_{HC}, using the numbering system of Dorrington et al. Particularly preferred positions at which to substitute or insert a cross-linking amino acid include, but are not limited to, amino acid position 2, 3, 4, 5, 6, 7, 8 or 9 of SEQ ID NO:11. While these position numbers refer to amino acid positions in SEQ ID NO:11, it should be noted that these amino acids are commonly referred to as, and correspond to, amino acids A329, D330, S331, N332, P333, R334, G335 and V336 of full-length IgE_{HC}, using the numbering system of Dorrington et al., 1978, *Immunol Rev* 41, 3-25. The same mutations could be made at the corresponding amino acids in the

full-length IgE_{HC} or within corresponding positions in IgE Fc-region fragments either alone or contained within other proteins.

A particularly useful type of modification is the introduction of a cysteine residue or a methionine residue into an IgE_{HC}. Such an introduction can result from an insertion 5 of an additional amino acid residue (i.e. cysteine or methionine) into the sequence or from the conversion of an existing amino acid residue into a cysteine or a methionine.

Methods of producing such mutants are known to those skilled in the art. One such method is through the manipulation of an IgE_{HC} nucleic acid molecule (e.g. by nucleotide insertions, deletions and/or substitutions) to produce a nucleic acid molecule encoding a 10 desired mutant IgE_{HC}. Nucleic acid molecules can be modified using a variety of techniques known to one skilled in the art such as, for example, site-directed mutagenesis, chemical treatment, restriction enzyme cleavage, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification, PCR mutagenesis, synthesis of oligonucleotide mixtures and ligation of mixture groups to "build" a mixture of nucleic 15 acid molecules, in-vitro or directed evolution and combinations thereof. The use of such techniques are known to those skilled in the art; see for example, Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press which is incorporated herein by reference in its entirety.

In one embodiment a mutant IgE_{HC} comprises an amino acid sequence at least 20 about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98% or at least about 100% identical to SEQ ID NO:11, but wherein the amino acid residue in said IgE_{HC} corresponding to position 2, 3, 4, 5, 6, 7, 8, or 9 of SEQ ID NO:11 is a cysteine or a methionine. In one embodiment, an IgE_{HC} of the present invention comprises an amino acid sequence at least about 80%, at least about 85%, at least about 25 90%, at least about 95%, at least about 98% or at least about 100% identical to an amino acid sequence selected from the group consisting of:

- (a) SEQ ID NO:13, wherein the amino acid residue in said IgE_{HC} corresponding to position 2 of SEQ ID NO:13 is a cysteine or a methionine;
- (b) SEQ ID NO:15, wherein the amino acid residue in said IgE_{HC} corresponding to 30 position 3 of SEQ ID NO:15 is a cysteine or a methionine;
- (c) SEQ ID NO:17, wherein the amino acid residue in said IgE_{HC} corresponding to position 4 of SEQ ID NO:17 is a cysteine or a methionine;

(d) SEQ ID NO:19, wherein the amino acid residue in said IgE_{HC} corresponding to position 5 of SEQ ID NO:19 is a cysteine or a methionine;

(e) SEQ ID NO:21, wherein the amino acid residue in said IgE_{HC} corresponding to position 6 of SEQ ID NO:21 is a cysteine or a methionine;

5 (f) SEQ ID NO:23, wherein the amino acid residue in said IgE_{HC} corresponding to position 7 of SEQ ID NO:23 is a cysteine or a methionine;

(g) SEQ ID NO:25, wherein the amino acid residue in said IgE_{HC} corresponding to position 8 of SEQ ID NO:25 is a cysteine or a methionine; and

(h) SEQ ID NO:27, wherein the amino acid residue in said IgE_{HC} corresponding to 10 position 9 of SEQ ID NO:27 is a cysteine or a methionine. Particularly useful IgE_{HC}'s of the present invention are IgE_{HC}'s comprising an amino acid sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31, SEQ ID NO:33, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:43, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49.

One embodiment of the present invention is a method to produce an IgE protein that is constrained in the closed conformation, said method comprising: (a) altering the nucleic acid sequence of a nucleic acid molecule encoding an unmodified IgE_{HC}; and (b) using the modified IgE_{HC} nucleic acid molecule to produce a mutant IgE protein 20 constrained in the closed conformation. In a preferred embodiment, the nucleic acid sequence is modified so that one or more cysteine residues are introduced into the IgE_{HC} protein. In a more preferred embodiment, the nucleic acid sequence is altered to encode a protein which has cysteine residues in a position corresponding to position 2, 3, 4, 5, 6, 7, 8 and/or 9 of SEQ ID NO:11.

25 The present invention also provides isolated mutant IgE_{HC} proteins lacking one or more N-linked glycosylation sites. The human IgE_{HC} is known to have at least three potential N-linked glycosylation sites. For example, the human IgE_{HC} can potentially be glycosylated at amino acids 44, 56 and/or 67 of SEQ ID NO:11, which correspond to amino acids 371, 373 and 394 of the full-length IgE using the numbering system of 30 Dorrington et al., 1978, *Immunol Rev* 41, 3-25. A useful embodiment of the present invention is an isolated mutant IgE_{HC} in which the sequence of one or more of the potential N-linked glycosylation sites have been modified such that the cellular glycosylation mechanism does not attach a carbohydrate moiety at that site. Suitable

modifications include amino acid substitutions, deletions and additions. Methods of making such modifications are known to those skilled in the art and include, for example, altering a nucleic acid sequence encoding an IgE_{HC}. A useful modification to make is one that modifies one or more amino acids from positions 44 through 46 of SEQ ID NO:11 and/or positions 56 through 58 of SEQ ID NO:11. A particularly useful embodiment is an isolated mutant IgE_{HC} in which the asparagine at position 44 of SEQ ID NO:11 and/or the asparagine at position 56 of SEQ ID NO:11 has been replaced by an amino acid other than an asparagine. Suitable embodiments include isolated mutant IgE_{HC}'s comprising an amino acid sequence at least about 85%, at least about 90%, at least about 95%, at least 10 about 98%, or at least about 100% identical to SEQ ID NO:11, but wherein the amino acid at position 44 and/or 56 of SEQ ID NO:11 is not an asparagine. A particularly suitable embodiment is an isolated mutant IgE_{HC} comprising the amino acid sequence of SEQ ID NO:8. Also contemplated are mutant IgE proteins comprising a mutant IgE_{HC} in which one or more glycosylation sites have been altered to prevent glycosylation at the modified site.

The present invention also provides 3-dimensional models of mutant IgE proteins comprising a mutant IgE_{HC} in which one or more glycosylation sites have been altered to prevent glycosylation at the modified site. Such models can describe a mutant IgE protein in the open or closed conformation and can be used to design or find compounds 20 that inhibit the binding of IgE to an Fc ϵ RI or Fc ϵ RI α .

One embodiment of the present invention is a nucleic acid molecule that encodes a protein of the present invention. Useful nucleic acid molecules include those that encode an IgE_{HC} comprising an amino acid sequence at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98% or at least about 100% 25 identical to SEQ ID NO:11, but wherein the amino acid residue in said IgE_{HC} corresponding to position 2, 3, 4, 5, 6, 7, 8, or 9 of SEQ ID NO:11 is a cysteine or a methionine. Particularly useful nucleic acid molecules include those that encode a mutant IgE_{HC} comprising an amino acid sequence at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98% or at least about 100% identical to an 30 amino acid sequence selected from the group consisting of:

(a) SEQ ID NO:13, wherein the amino acid residue in said IgE_{HC} corresponding to position 2 of SEQ ID NO:13 is a cysteine or a methionine;

- (b) SEQ ID NO:15, wherein the amino acid residue in said IgE_{HC} corresponding to position 3 of SEQ ID NO:15 is a cysteine or a methionine;
- (c) SEQ ID NO:17, wherein the amino acid residue in said IgE_{HC} corresponding to position 4 of SEQ ID NO:17 is a cysteine or a methionine;
- 5 (d) SEQ ID NO:19, wherein the amino acid residue in said IgE_{HC} corresponding to position 5 of SEQ ID NO:19 is a cysteine or a methionine;
- (e) SEQ ID NO:21, wherein the amino acid residue in said IgE_{HC} corresponding to position 6 of SEQ ID NO:21 is a cysteine or a methionine;
- (f) SEQ ID NO:23, wherein the amino acid residue in said IgE_{HC} corresponding to 10 position 7 of SEQ ID NO:23 is a cysteine or a methionine;
- (g) SEQ ID NO:25, wherein the amino acid residue in said IgE_{HC} corresponding to position 8 of SEQ ID NO:25 is a cysteine or a methionine; and
- (h) SEQ ID NO:27, wherein the amino acid residue in said IgE_{HC} corresponding to position 9 of SEQ ID NO:27 is a cysteine or a methionine.

15 Preferred nucleic acid molecules are those that encode an IgE_{HC} comprising an amino acid sequence at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98% or at least about 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID 20 NO:23, SEQ ID NO:25 and SEQ ID NO:27.

Nucleic acid molecules useful in the present invention can be produced by, for example, recombinant nucleic acid technology or by chemical synthesis. A nucleic acid molecule of the present invention can be DNA, RNA, or a derivative of DNA and RNA. Nucleic acid molecules of the present invention include natural forms, including allelic 25 variants, nucleic acid molecules optimized for expression in a particular host and other nucleic acid molecules modified by nucleotide insertions, deletions, substitutions and/or inversions. A useful nucleic acid molecule of the present invention is a nucleic acid molecule comprising a nucleic acid sequence at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least 30 about 100% identical in sequence to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24 or SEQ ID NO:26, wherein the nucleic acid sequence encodes a protein that

elicits an immune response to an IgE_{HC} protein that comprises the amino acid sequence of SEQ ID NO:11.

One embodiment of the present invention is a nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

- 5 (a) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 2 of SEQ ID NO:10 encodes a cysteine or a methionine;
- 10 (b) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 3 of SEQ ID NO:10 encodes a cysteine or a methionine;
- 15 (c) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 4 of SEQ ID NO:10 encodes a cysteine or a methionine;
- 20 (d) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 5 of SEQ ID NO:10 encodes a cysteine or a methionine;
- 25 (e) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 6 of SEQ ID NO:10 encodes a cysteine or a methionine;
- 30 (f) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 7 of SEQ ID NO:10 encodes a cysteine or a methionine;
- 35 (g) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 8 of SEQ ID NO:10 encodes a cysteine or a methionine; and

(h) a nucleic acid sequence at least about 90%, at least about 95%, at least about 98%, at least about 100% identical to SEQ ID NO:10, wherein the codon in said nucleic acid sequence corresponding to the codon at position 9 of SEQ ID NO:10 encodes a cysteine or a methionine.

5 It should be noted that it is well known by those skilled in the art a codon consists of 3 nucleotides and that references to a codon position in a SEQ ID NO. refers to the 3 nucleotides making that make up that codon. For example, reference to the codon at position 2 of SEQ ID NO:10 refers to nucleotides 4-6 of SEQ ID NO:10.

A preferred nucleic acid molecule is one having the above characteristics and which
10 encodes a protein having functions including, but not limited to antigen binding, eliciting an immune response to an IgE protein comprising an IgE_{HC} that comprises an amino acid sequence of SEQ ID NO:11 and binding an antibody generated to an IgE protein comprising an IgE_{HC} that comprises an amino acid sequence of SEQ ID NO:11.

A suitable nucleic acid molecule of the present invention is a nucleic acid
15 molecule comprising a nucleic acid sequence selected from SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48.

One embodiment of the present invention is a recombinant molecule that comprises a nucleic acid molecule operatively linked to a transcriptional control sequence. The phrase operatively linked refers to joining of a nucleic acid molecule to a transcription control sequence in a manner such that the molecule is able to be expressed
25 when transformed into a host organism. A suitable host organism is any organism capable of directing expression from a nucleic acid molecule of the present invention. As used herein, an expression vector is a DNA or RNA vector, typically either a plasmid or viral genome, that is capable of transforming a cell and of effecting expression of a specified nucleic acid molecule. A preferred recombinant molecule of the present
30 invention contains regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant microorganism and that control the expression of nucleic acid molecules of the present invention. Transcription control sequences are

sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant microorganisms of the present invention. A variety of such transcription control sequences are known to those skilled in the art; examples included, but are not limited to, *tac*, *lac*, *trp*, *trc*, oxy-pro, *omp/lpp*, *rrnB*, bacteriophage lambda (such as lambda *p_L*, also referred to herein as lambda PL) and lambda *p_R* (also referred to herein as lambda PR) and fusions that include such promoters), bacteriophage T7, T7*lac*, bacteriophage T3, bacteriophage SP6, SP01, alpha-mating factor, alcohol oxidase (AOX), antibiotic resistance gene, and other sequences capable of controlling gene expression in *E. coli*, methyltrophic yeast microorganisms, insect cells or other cells useful for expressing proteins; it is to be noted that this list is not intended to be limiting as many additional transcriptional control sequences are known.

15 A preferred recombinant molecule includes a nucleic acid molecule that encodes an IgE_{HC} of the present invention, operatively linked to an insect cell promoter.

Another embodiment of the present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a host cell. 20 Such a recombinant vector contains heterologous nucleic acid sequences, that is nucleic acid sequences that are not naturally found adjacent to nucleic acid molecules of the present invention and that preferably are derived from a species other than the species from which the nucleic acid molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

25 Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulation of IgE nucleic acid molecules of the present invention.

One embodiment of the present invention is a recombinant cell, which is a host cell transformed with a nucleic acid molecule of the present invention. A preferred recombinant molecule comprises a recombinant molecule of the present invention.

30 Recombinant DNA technologies can be used to improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are

translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein.

One embodiment of an IgE_{HC} of the present invention is a fusion protein. Suitable fusion segments for use with the present invention include, but are not limited to, segments that can: link two or more IgE_{HC} proteins of the present invention to form multimers; enhance a protein's stability; facilitate the purification of an IgE_{HC} protein; and/or to affect the immune response to an IgE or IgE_{HC} protein. A suitable fusion segment can be a domain of any size that has the desired function (e.g., imparts increased stability, imparts increased immunogenicity to a protein, and/or simplifies purification of a protein). Fusion segments can be joined to amino and/or carboxyl termini of an IgE_{HC} of the present invention and can be susceptible to cleavage in order to enable straight-forward recovery of such protein. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid molecule that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of an IgE_{HC} protein. Preferred fusion segments include a metal binding domain (e.g., a poly-histidine segment); an immunoglobulin binding domain (e.g., Protein A; Protein G; T cell; B cell; Fc receptor or complement protein antibody-binding domains); a sugar binding domain (e.g., a maltose binding domain); and/or a "tag" domain (e.g., at least a portion of β-galactosidase, a strep tag peptide, a T7 tag peptide, a Flag™ peptide, or other domains that can be purified using compounds that bind to the domain, such as monoclonal antibodies). More preferred fusion segments include metal

binding domains, such as a poly-histidine segment; a maltose binding domain; a strep tag peptide, such as that available from Biometra in Tampa, FL; and an S10 peptide.

Effective culturing conditions to produce a mutant IgE_{HC} or a mutant IgE protein of the present invention include, but are not limited to, effective media, bioreactor, 5 temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce an a mutant IgE_{HC} or a mutant IgE protein of the present invention. Such a medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Recombinant 10 cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for yeast, *E. coli*, insect cells or other cells suitable for culture. Determining such culturing conditions are within the expertise of one of ordinary skill in the art.

15 Depending on the vector and host system used for production, resultant proteins of the present invention may either remain within the recombinant cell; be secreted into the culturing, or fermentation, medium; or be secreted into a space between two cellular membranes. In a preferred embodiment, the protein is in the medium and, hence, can be easily separated from the cell.

20 Proteins of the present invention can be purified using a variety of purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization. Proteins of the present 25 invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein as a diagnostic, therapeutic or prophylactic, or as a screening tool.

The ability of IgE_{HC} or IgE proteins of the present invention as well as of unmodified IgE_{HC} or IgE proteins to selectively bind to Fc_εRI or Fc_εRIα protein can be 30 assayed by methods known in the art, such as, but not limited to, those disclosed herein and in PCT Publication No. WO 00/26246, Jardetzky et al., published May 11, 2000; U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published January 2, 2003; PCT Publication No. WO 01/69253 A3, Jardetzky et al., published September 20, 2001;

U.S. Patent Publication No. 20010039479, Jardetzky et al., published November 8, 2001; and PCT Publication No. WO 01/68861 A3, Jardetzky et al., published September 20, 2001. As used herein, the term, selectively binds to Fc ϵ RI or to Fc ϵ RI α protein refers to the ability of a protein to preferentially bind to Fc ϵ RI or Fc ϵ RI α protein, without being able to substantially bind to other FcR proteins. Preferably, an Fc ϵ RI or Fc ϵ RI α binds to an IgE protein with an affinity (K_A) of at least about 10^8 liters/mole (M^{-1}), more preferably of at least about $10^9 M^{-1}$, and even more preferably of at least about $10^{10} M^{-1}$. Methods to compare binding activity of the mutant proteins of the present invention with the binding activity of unmodified proteins are also known in the art and include, but are not limited to, those methods disclosed herein and in PCT Publication No. WO 00/26246, Jardetzky et al., published May 11, 2000; U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published January 2, 2003; PCT Publication No. WO 01/69253 A3, Jardetzky et al., published September 20, 2001; U.S. Patent Publication No. 20010039479, Jardetzky et al., published November 8, 2001; and PCT Publication No. 10 WO 01/68861 A3, Jardetzky et al., published September 20, 2001.

The present invention also includes a method to identify a compound that binds to an IgE protein, thereby placing, restraining, maintaining or stabilizing that IgE protein in a closed form (i.e., such compound binds to an IgE protein either in or resulting in a closed conformation). Preferably such a compound inhibits an IgE protein from binding to Fc ϵ RI. It is to be appreciated that methods to identify such a compound can utilize unmodified or mutant IgE_{HC} or IgE proteins of the present invention. As such, although the methods discussed below may cite the use of a mutant IgE protein, it is to be appreciated that mutant IgE_{HC} proteins of the present invention can also be used.

One embodiment is a method to identify a compound that binds the closed form of an IgE protein, said method comprising: (a) contacting a mutant IgE protein constrained in the closed form with a candidate compound; and (b) determining if such candidate compound binds to the IgE closed form mutant. In a preferred embodiment, such candidate compound, assuming it bound to the closed form of an IgE protein, is then contacted with IgE and an Fc ϵ RI or Fc ϵ RI α protein to determine whether such candidate compound inhibits binding between IgE and such Fc ϵ RI or Fc ϵ RI α protein. In another preferred embodiment, such candidate compound, assuming it bound to a closed form of an IgE protein, is also contacted with an IgE protein in the open conformation to determine if the candidate compound binds to IgE in the open conformation. Another

embodiment is a method to identify a compound that inhibits the binding of IgE to Fc ϵ RI by a method comprising: (a) contacting a mutant IgE protein constrained in the closed form with a candidate compound in the presence of a Fc ϵ RI or Fc ϵ RI α protein; and (b) determining if such candidate compound inhibits binding of the IgE protein to the Fc ϵ RI or Fc ϵ RI α protein.

A preferred compound is one that selectively binds an IgE protein in the closed conformation. As used herein, the term, selectively binds to the closed conformation of an IgE protein, refers to the ability of a compound to preferentially bind to the closed conformation of an IgE protein, without being able to substantially bind to other proteins or molecules, including an IgE protein in the open conformation. A compound that is capable of selectively binding to an IgE protein is also referred to herein as an IgE-binding compound. A particularly preferred compound is a compound that binds to an IgE protein in the closed conformation, but not in the open conformation (except to cause such IgE to adopt a closed conformation) and that inhibits the binding of an IgE protein to Fc ϵ RI or Fc ϵ RI α .

Suitable binding assays are known to those skilled in the art and are conducted under conditions suitable to form a binding complex; such conditions, e.g., appropriate concentrations, buffers, temperatures, reaction times, as well as methods to optimize such conditions are known to those skilled in the art. See, for example Wingfield, et al., 1996, Current Protocols in Protein Science, Volume 1, John Wiley and Sons Publisher, which is incorporated herein in its entirety by reference, Sambrook et al., *ibid.*, PCT Publication No. WO 00/26246, Jardetzky et al., published May 11, 2000; U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published January 2, 2003; PCT Publication No. WO 01/69253 A3, Jardetzky et al., published September 20, 2001; U.S. Patent Publication No. 20010039479, Jardetzky et al., published November 8, 2001; and PCT Publication No. WO 01/68861 A3, Jardetzky et al., published September 20, 2001, and Example 1.

Examples of assays useful for detecting binding of a compound to an IgE protein include, but are not limited to, an enzyme-linked immunoassay, a radioimmunoassay, a fluorescence immunoassay, a chemiluminescent assay, a lateral flow assay, an agglutination assay, a particulate-based assay, e.g., using particulates such as, but not limited to, magnetic particles or plastic polymers, such as latex or polystyrene beads, an immunoprecipitation assay, a BioCore™ assay, e.g., using colloidal gold, and an immunoblotting assay, e.g., a western blot. Such assays are well known to those skilled

in the art. Assays can be used to give qualitative or quantitative results depending on how they are used. Some assays, such as agglutination, particulate separation, and immunoprecipitation, can be observed visually, e.g., either by eye or by a machine, such as a densitometer or spectrophotometer, without the need for a detectable marker. In other assays, conjugation, i.e., attachment, of a detectable marker to one of the binding components of the assay aids in detecting complex formation. Examples of detectable markers include, but are not limited to, a radioactive label, an enzyme, a fluorescent label, a chemiluminescent label, a chromophoric label or a ligand. A ligand refers to a molecule that binds selectively to another molecule. Preferred detectable markers include, but are not limited to, fluorescein, a radioisotope, a phosphatase, e.g., alkaline phosphatase, biotin, avidin, a peroxidase, e.g., horseradish peroxidase, and biotin-related compounds or avidin-related compounds, e.g., streptavidin or ImmunoPure® NeutrAvidin available from Pierce, Rockford, IL. Such methods can be used to test one or more compounds at a time. Preferred methods test more than one compound at the same time and can include screening methods to analyze many candidate compounds simultaneously. It is within the ability of one skilled in the art to optimize such assays to determine which compounds bind an IgE protein in the closed conformation, which compounds bind an IgE protein in the open conformation, which compounds inhibit an IgE protein from binding to its receptor, which compounds do not bind an IgE protein in the closed conformation, and which compounds do not bind an IgE protein in the open conformation.

Suitable compounds that bind an IgE protein in the closed conformation can be naturally produced compounds or synthetically produced compounds and include proteins, carbohydrates, organic molecules, substrate analogs, and other large or small molecules. Such compounds may be identified, for example, by screening chemical libraries or phage display libraries and the like.

In one embodiment, a mixture of candidate compounds is applied to a column to which IgE protein in the open conformation has been immobilized. Compounds which flow through the column (those that do not bind IgE in the open conformation) are collected and applied to a second column to which IgE in the closed conformation has been immobilized. Compounds that do not bind this form of the IgE protein will flow through the column. Compounds which do bind IgE in the closed conformation are then eluted from the column for further analysis.

The present invention also includes a therapeutic composition comprising at least one IgE-binding compound of the present invention, a protein of the present invention or some combination thereof. A preferred compound to include in a therapeutic composition is one which binds the closed form of IgE and/or when bound to IgE restraints the IgE from existing in an open conformation, thereby inhibiting the IgE from binding to a Fc ϵ RI. A particularly preferred compound to include in a therapeutic composition is one which inhibits binding of IgE to a Fc ϵ RI. Therapeutic compositions of the present invention can be formulated in an excipient that the animal to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions.

Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, or o-cresol, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

In one embodiment of the present invention, a therapeutic composition can include a carrier. Carriers include compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release vehicles, biodegradable implants, liposomes, bacteria, viruses, other cells, oils, esters, and glycols.

One embodiment of the present invention is a controlled release formulation that is capable of slowly releasing a composition of the present invention into an animal. As used herein, a controlled release formulation comprises a composition of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Other controlled release

formulations of the present invention include liquids that, upon administration to an animal, form a solid or a gel *in situ*. Preferred controlled release formulations are biodegradable, i.e., bioerodible.

In one embodiment, a therapeutic composition of the present invention can be used to protect an animal from a disease mediated by IgE by administering such composition to such animal in order to prevent undesirable effects of IgE. An example of an IgE-mediated disease is an allergy. In one embodiment, the therapeutic composition of the present invention is administered to reduce the activity and or amount of IgE in an animal. Such administration can include, but is not limited to, oral, intravenous, intramuscular, intra ocular, mucosal, intranasal, subcutaneous, or transdermal application. A preferred route of administration is subcutaneous. In order to protect an animal from a disease mediated by IgE, a therapeutic composition of the present invention is administered to the animal in an effective manner such that the composition is capable of protecting that animal from a disease mediated by IgE. Therapeutic compositions of the present invention can be administered to animals prior to disease in order to prevent disease and/or can be administered to animals after disease occurs. The exact dose, administration regimen, and administration route of therapeutic compositions of the present invention can be determined by one skilled in the art. Additional teachings are provided, for example, in PCT Publication No. WO 00/26246, Jardetzky et al., published May 11, 2000; U.S. Patent Publication No. 20030003502-A1, Jardetzky et al., published January 2, 2003; PCT Publication No. WO 01/69253 A3, Jardetzky et al., published September 20, 2001; U.S. Patent Publication No. 20010039479, Jardetzky et al., published November 8, 2001; and PCT Publication No. WO 01/68861 A3, Jardetzky et al., published September 20, 2001.

One embodiment of the present invention is a kit comprising a mutant IgE_{HC} or IgE protein of the present invention. Such a kit is used to identify a compound that binds to IgE protein, preferably IgE protein in the closed conformation or that when such compound binds to IgE, prevents or restrains it from maintaining or achieving an open conformation. Such a kit may also comprise tubes, bottles, reagents, syringes, literature, packaging and the like.

The following example is provided for the purpose of illustration and is not intended to limit the scope of the present invention. The following example includes a

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number of recombinant DNA and protein chemistry techniques known to those skilled in the art; see for example Sambrook et al., *ibid.*

Example 1

This Example describes the construction and binding abilities of several cysteine 5 mutants of IgE_{HC} proteins or IgE_{HC}'s

A. Construction of IgE_{HC} cysteine mutants:

Several mutant IgE_{HC} proteins were created by substituting a cysteine into an unmodified IgE_{HC} protein having SEQ ID NO:11 thereby enabling the potential formation of interchain disulfide bonds. The mutant proteins were created using 10 polymerase chain reaction (PCR) mutagenesis as follows:

Using an unmodified (unmod) IgE_{HC} nucleic acid molecule having SEQ ID NO:10 as a template, mutant IgE_{HC} nucleic acid molecules were amplified by PCR, under standard conditions, using a 5' primer that introduced mutations creating a cysteine residue at various position. The 5' primers are as follows:

15 C328A 5'T AGG GCG GAT CCC GCT GCA GAT TCG AAC CCG AGA GGG GTG AGC G 3'
(SEQ ID NO:28)
Arg Ala Asp Pro Ala Ala Asp Ser Asn Pro Arg Gly Val Ser
(SEQ ID NO:29)

20 C328S 5'T AGG GCG GAT CCC TCT GCA GAT TCG AAC CCG AGA GGG GTG AGC G 3'
(SEQ ID NO:30)
Arg Ala Asp Pro Ser Ala Asp Ser Asn Pro Arg Gly Val Ser
(SEQ ID NO:31)

25 C328 5'T AGG GCG GAT CCC TGT GCG GAT TCG AAC CCG AGA GGG GTG AG 3'
(SEQ ID NO:32)
Arg Ala Asp Pro Cys Ala Asp Ser Asn Pro Arg Gly Val
(SEQ ID NO:33)

30 C329 5'T AGG GCG GAT CCC gcg tgt GAT TCG AAC CCG AGA GGG GTG AG 3'
(SEQ ID NO:34)
Arg Ala Asp Pro Ala Cys Asp Ser Asn Pro Arg Gly Val
(SEQ ID NO:35)

35 C330 5'T AGG GCG GAT CCC gcg GCG tgt TCG AAC CCG AGA GGG GTG AG 3'
(SEQ ID NO:36)
Arg Ala Asp Pro Ala Ala Cys Ser Asn Pro Arg Gly Val
(SEQ ID NO:37)

40 C331 5'T AGG GCG GAT CCC gcg GCG GAT tgt AAC CCG AGA GGG GTG AG 3'
(SEQ ID NO:38)
Arg Ala Asp Pro Ala Ala Asp Cys Asn Pro Arg Gly Val
(SEQ ID NO:28)

45 C332 5'T AGG GCG GAT CCC gcg GCG GAT TCG tgt CCG AGA GGG GTG AG 3'
(SEQ ID NO:39)
Arg Ala Asp Pro Ala Ala Asp Ser Cys Pro Arg Gly Val
(SEQ ID NO:40)

C333 5'T AGG GCG GAT CCC gcg GCG GAT TCG AAC tgt AGA GGG GTG AG 3'
(SEQ ID NO:41)

Arg Ala Asp Pro Ala Ala Asp Ser Asn Cys Arg Gly Val
5 (SEQ ID NO:42)

C334 5'T AGG GCG GAT CCC gcg GCG GAT TCG AAC CCG tgt GGG GTG AG 3'
(SEQ ID NO:43)

Arg Ala Asp Pro Ala Ala Asp Ser Asn Pro Cys Gly Val
10 (SEQ ID NO:44)

C335 5'T AGG GCG GAT CCC gcg GCG GAT TCG AAC CCG AGA tgt GTG AG 3'
(SEQ ID NO:45)

Arg Ala Asp Pro Ala Ala Asp Ser Asn Pro Arg Cys Val
15 (SEQ ID NO:46)

C336 5'T AGG GCG GAT CCC gcg GCG GAT TCG AAC CCG AGA GGG tgt AG 3'
(SEQ ID NO:47)

Arg Ala Asp Pro Ala Ala Asp Ser Asn Pro Arg Gly Cys
20 (SEQ ID NO:48)

Each of the mutant IgE_{HC} nucleic acid molecules was ligated into the expression vector pAcGP67A (Catalog Number 22122OP, Becton Dickinson Pharmingen, Franklin Lakes, N.J.). The encoded sequence of the N-terminus of the mature (signal sequence cleaved) protein is ADPCAD with C corresponding to C328 of the mature IgE using the numbering according to Dorrington and Bennich, 1978, *Immunol Rev* 41, 3-25. The plasmids were transformed into insect (HI-5) insect cells using standard protocols, expressed and the resulting proteins analyzed using western blot analysis.

B. Western-blot analysis of unmodified and mutant IgE_{HC}'s

Following expression in insect cells, the IgE_{HC} proteins were analyzed by western-blot analysis. Samples of infected cell supernatants were added to PAGE sample buffer either with or without reducing agent (e.g. 5 mM DTT). The samples were then boiled and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following separation, the proteins were transferred to Immobilon membranes and the proteins visualized using an alkaline-phosphatase conjugated, goat anti-human polyclonal anti-IgE antibody (Catalog 075-1004, Kirkegaard & Perry Labs, Gaithersburg, MD) using standard western blotting protocol. The results of this analysis are shown in Figure 3.

C. Ability of mutant IgE_{HC} proteins to bind Fc ϵ RI α

The ability of the unmodified and mutant IgE_{HC} proteins to bind the Fc ϵ RI α protein was compared using an ELISA assay as follows:

The wells of a microtiter plate were coated (50 ng/well) with human Fc ϵ RI α (produced as described in U.S. Patent Publication No. US-2001-0039479-A1) in PBS and the plate incubated overnight at 4°C. The plate was washed 3X with WASH buffer (0.5%

Tween-20 in 10 mM Tris, pH 7.5, 150 mM NaCL) and blocked for one hour at room temperature (RT) using BLOCK buffer (5% dried milk in 10 mM Tris, pH 7.5, 150 mM NaCL). The plate was again washed 3X with WASH buffer and 100 µl of IgE_{HC} (either unmodified IgE_{HC} or mutant IgE_{HC} diluted from 0.5 to 0.065 in BLOCK buffer) was added to various wells of the plate. To half of the samples was added 5 mM DTT. The plate was then incubated for one hour at RT, washed 3X with WASH buffer, and 100 µl of alkaline-phosphatase conjugated, goat anti-human polyclonal anti-IgE antibody (Catalog 075-1004, Kirkegaard & Perry Labs, Gaithersburg, MD)(use a 1:1000 dilution of stock solution) was added to each well. The plate was incubated at RT for one hour, washed 3X with WASH buffer, rinsed three times with water, and 50 µl of p-nitrophenyl phosphate (PNPP) was added to each well. The plate was incubated for 30-60 minutes at RT and the reaction was stopped by the addition of 10 µl of 0.5M EDTA. The plate was read at 450 nM using an ELISA plate reader. The results are shown below in Table 1.

Table 1.

		C328 (unmod)		C329		C330		C331	
		5 mM DTT		5 mM DTT		5 mM DTT		5 mM DTT	
Dilution		+	-	+	-	+	-	+	-
0.5		0.224	0.257	0.14	0.257	0.159	0.161	0.125	0.059
0.25		0.19	0.265	0.0155	0.192	0.13	0.13	0.102	0.038
0.125		0.106	0.243	0	0.065	0.039	0.075	0.0895	0.013
0.065		0.0115	0.195	-0.003	0.022	0.001	0.062	0.066	0.038
		C332		C333		C334		C335	
		5 mM DTT		5 mM DTT		5 mM DTT		5 mM DTT	
Dilution		+	-	+	-	+	-	+	-
0.5		0.11	0.032	0.1625	0.109	0.0185	-0.004	0.003	-0.003
0.25		0.0185	0.012	0.126	0.021	0.002	-0.0075	-0.0025	-0.0075
0.125		0.006	-0.0045	0.12	-0.01	-0.0035	-0.015	-0.006	-0.014
0.125		0.002	0.001	0.068	0.003	-0.006	-0.0055	-0.0075	-0.0075
		C336							
		5 mM DTT							
Dilution		+	-						
0.5		0.0705	0.039						
0.25		0.028	0.014						
0.125		0.003	-0.0195						
0.125		-0.006	-0.003						

15

These data indicate that IgE_{HC} proteins that have cysteine residues substituted into their sequence, thereby allowing the formation of inter-chain disulfide bonds, have a reduced ability to bind to the human Fc ϵ RI α protein.

Example 2.

This Example describes the production and analysis of crystals of a glycosylation mutant of the Ce3/Ce4 domain of human IgE.

A. Construction of a IgE Fc-Ce3/Ce4 CHO mutant

5 A new mutant IgE-Fc protein was created in which the sequence of the glycosylation sites in the IgE-Fc-region Ce3/Ce4 domain protein were altered in order to prevent glycosylation at these sites. The CHO mutant was created using polymerase chain reaction (PCR) mutagenesis as follows:

Using a nucleic acid molecule encoding the IgE Ce3/Ce4 domains of IgE (SEQ ID 10 NO:10) as a template, 5' nucleic acid molecule and 3' nucleic acid molecule were created in two separate PCR reactions. The 5' nucleic acid molecule was synthesized using the primers HIGEFC1b and N371Q. The primer HIGEFC1b, which has the sequence 5' TAGGGCTACGTAGATTCCAACCCGAGAGG 3', (represented by SEQ ID NO:3) contains a SnaB I restriction site and encodes an a portion of the Fc protein having an N-terminal sequence (following restriction digest with Sna BI) of VDSNPR with D corresponding to D330 of the mature sequence. The primer N371Q, which has the sequence 5' ACTGGCTCGAGACCAGGTAGCTGCACGGTCCCCTTGCTGGGT 3' (represented by SEQ ID NO:4), introduces unique Xho I and Pvu II sites and contains the mutation which changes the asparagine at position 371 to a glutamine. The 3' nucleic 15 acid molecule was synthesized using primers N383Q and HIGEFC2B. Primer N383Q, which has the sequence 5'
CCTGGTCTCGAGCCAGTGGGAAGCCTGTGCAACACTCCACCAGAAAGGAGGA
G 3' (represented by SEQ ID NO:5), introduces a unique Xho I restriction site and contains the mutation that changes the asparagine at position 383 to a glutamine. Primer 20 HIGEFC2B, which has the sequence 5'
TCTAGGCAGCGGCCGCTTATCATTTACCGGGATTACAG 3' (represented by SEQ ID NO:6), terminates the Fc sequence at Lys 547 and contains a Not I restriction site.
The 5' and 3' nucleic acid molecules were generated using standard PCR conditions, gel purified and then digested with the restriction enzymes SnaB I and Xho I (5' fragment) or 25 Not I and Xho I (3'fragment). The digested nucleic acid molecules were then ligated together at their Xho I sites to yield a nucleic acid molecule encoding the Ce3/Ce4 domains containing the glycosylation site mutations (represented by SEQ ID NO:7).
Translation of the newly constructed nucleic acid molecule results in a protein

(represented by SEQ ID NO:8) lacking glycosylation recognition sites at positions 371 and 383.

For expression in insect cells, the newly constructed nucleic acid molecule encoding the C ϵ 3/C ϵ 4 domains for the carbohydrate mutant was amplified using primers 5 IgECABac and HIGEF2b. The primer IgECABac, which has the sequence 5' TAGGGCGGATCCCTGTGCAGATTGAAACCCGAGAGGGGTGAGCG 3' (represented by SEQ ID NO:9), contains a BamH I site for cloning the nucleic acid molecule behind a signal sequence in the expression vector pAcGP67A (Catalog Number 22122OP, Becton Dickinson Pharmingen, Franklin Lakes, N.J.). The encoded sequence 10 of the N-terminus of the mature (signal sequence cleaved) protein is ADPCAD with C corresponding to C328 of the mature IgE. Following amplification, the nucleic acid molecule was digested with BamH I and Not I, gel purified and ligated into the pAcGP67A vector.

B. Expression, purification and crystallization of the CHO mutant proteins 15 The IgE-Fc CHO mutant protein was expressed, purified to homogeneity and crystallized. The expression, purification, crystallization, characterization and data collection and refinement of the mutant protein was performed as described in Example 1 and Example 2 of U.S. Patent Publication No. US-2001-0039479-A. Three new crystals were generated and data obtained from these crystals are shown in Table 2.

20

Table 2. Data for the CHO mutant IgE Protein Crystals

	Space Group	a	b	c	β	Mol/asym	Resolution
Crystal 1	C2	158	108	50	102	1.5	2.3 Å
Crystal 2	P2 ₁	66	99	77	97	2.0	2.45 Å
Crystal 3	P2 ₁ (Big)	48	104	150	96	3.0	2.8 Å

The data collection and refinement statistics obtained from Crystal 1 are shown below in Table 3.

Statistics based on m10_bi50CHO_m25_bi5_m35_P+EBA.pdb

5

Table 3: Data Collection and Refinement

C2 (f5) (pH 4.6)	
Data Collection Statistics	
Source	APS DND 5-ID
Wavelength (Å)	1.008
Resolution (Å)	30.0-2.30 (2.38 - 2.30) [†]
Completeness	98.8% (89.8%) [†]
Unique reflections (Total)	36,675 (139,507)
Average redundancy	3.8 (3.7) [†]
<I/I>	15.4 (2.4) [†]
R _{merge}	6.2% (41.3%) [†]
Refinement	
No. of Reflections (free)	36,675 (1,866)
R _{work} /R _{free}	27.4/29.3
Atoms (Total)	5059
Protein Atoms	4909
Water Molecules	0
Carbohydrate Atoms	150
Average B factor	
Protein	*59.6 Å ²
RMS Deviations from Ideality	
Bond angles	1.33 °
Bond lengths	0.008 Å
Ramachandran (ΦΨ)	
Favored	86.5 %
Allowed	12.2 %
Generous	0.9 %
Disallowed	0.4 %

[†] Values for the highest resolution shell are shown in parentheses

R_{merge}= $\sum|I_i - \langle I \rangle| / \sum|I_i|$, where I_i is the intensity of an individual reflection and <I> is the average intensity of that reflection.

R_{work/free}= $\sum||F_p - |F_c|| / \sum|F_p|$, where F_c is the calculated and F_p is the observed structure factor amplitude. R_{work} and R_{free} were calculated using the working set and test set reflections, respectively.

Cell a=158.8 b=108.5 c= 50.4 β=102° [**These are Denzo ave (not scalepack)
refined]

Refinement values based on m10_bi50CHO_m25_bi5_m35_p+EBA.pdb

Total # residues = 627

10 1.5 dimers/asu (=1.5 Fc molecules/asu)

The data collection and refinement statistics obtained from Crystal 2 are shown below in Table 4.

Statistics based on min300_c3l.pdb

Table 4: Data Collection and Refinement

	<i>P2₁ (f8) (pH 4.6)</i>
Data Collection Statistics	
Source	APS DND 5-ID
Wavelength (Å)	1.000
Resolution (Å)	30.0-2.45 (2.54 - 2.45) [†]
Completeness	98.2% (82.3%) [†]
Unique reflections (Total)	36,017 (128,258)
Average redundancy	3.63 (2.3) [†]
$\langle I/\sigma_I \rangle$	22.2 (3.35) [†]
R _{merge}	5.9% (24.1%) [†]
Refinement	
No. of Reflections (free)	34,123 (1,824)
R _{work} /R _{free}	29.3/31.2
Atoms (Total)	6,345
Protein Atoms	6,345
Water Molecules	0
Carbohydrate Atoms	0
Average B factor	
Protein	48.3 Å²
RMS Deviations from Ideality	
Bond angles	3.06 °
Bond lengths	0.03 Å
Ramachandran (ϕ, ψ)	
Favored	85.7 %
Allowed	11.2 %
Generous	1.8 %
Disallowed	1.3 %

† Values for the highest resolution shell are shown in parentheses

$R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum |I_i|$, where I_i is the intensity of an individual reflection and $\langle I \rangle$ is the average intensity of that reflection.

$R_{\text{work/free}} = \sum |F_p - |F_c|| / \sum |F_p|$, where F_c is the calculated and F_p is the observed structure factor amplitude. R_{work} and R_{free} were calculated using the working set and test set reflections, respectively.

5 Cell a=65.7 b= 99.6 c=77.9 $\beta=97.1^\circ$
 Refinement values based on min300_c3l.pdb
 Total # residues = 816
 2 dimers/asu (= 2 Fc molecules/asu)

The data collection and refinement statistics obtained from Crystal 3 are shown below in Table 5.

Statistics based on m200_bi14v3_bg10_bd8_m250_BE_new.pdb

Table 5: Data Collection and Refinement

P2₁ "BIG" (f7) (pH 4.6)

Data Collection Statistics	
Source	APS DND 5-ID
Wavelength (Å)	1.000
Resolution (Å)	30.0-2.80 (2.90 - 2.80) [†]
Completeness	98.9% (100%) [†]
Unique reflections (Total)	36,906 (139,248)
Average redundancy	3.8 (3.8) [†]
$\langle I/\sigma_I \rangle$	16.7 (4.9) [†]
R _{merge}	7.3% (54.1%) [†]
Refinement	
No. of Reflections (free)	35,046 (1,860)
R _{work} /R _{free}	31.3/36.0
Atoms (Total)	9,300
Protein Atoms	9,300
Water Molecules	0
Carbohydrate Atoms	0
Average B factor	
Protein	72.3 Å ²
RMS Deviations from Ideality	
Bond angles	1.41 °
Bond lengths	0.009 Å
Ramachandran (ϕ, ψ)	
Favored	82.9 %
Allowed	13.9 %
Generous	2.5 %
Disallowed	0.6 %

[†] Values for the highest resolution shell are shown in parentheses

$R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum |I|$, where I_i is the intensity of an individual reflection and $\langle I \rangle$ is the average intensity of that reflection.

$R_{\text{work/free}} = \sum |F_p - |F_c|| / \sum |F_p|$, where F_c is the calculated and F_p is the observed structure factor amplitude. R_{work} and R_{free} were calculated using the working set and test set reflections, respectively.

5 Cell a=48.9 b=104.9 c=150.0 $\beta=96.2^\circ$
 Refinement values based on m165_bi15v2_bg10_bd28_m250_BE_new.pdb
 Total # residues = 1,182
 3 dimers/asu (= 3 Fc molecule/asu)

C. Description of the IgE Fc-region Ce3/Ce4 domain mutant protein structure

The new crystal forms reveal additional information on the IgE-Fc conformational change, showing that the C3 domains can adopt a variety of intermediate conformations between the open and closed forms. Interestingly, the C3 domains that are in the closed 5 conformation are generally more similar and therefore probably more restricted in their conformational flexibility. In contrast, those C3 domains that are closer to the open conformation adopt a wider variety of side-side conformations, suggesting that there are fewer restrictions on the movements of the domains in the open configuration. These differences may have an impact on drug design and binding to the different forms and 10 could be important for docking experiments.

Analysis of the conformational ensemble revealed by the determination of these additional crystal forms of the IgE-Fc suggest how dynamic motions within the IgE-Fc may be coupled to receptor binding and dissociation. For example, conformational changes of the IgE-Fc are likely important to the microscopic steps in association and 15 release from the receptor Binding Sites 1 and 2. In addition, analysis of the conformational ensemble suggests how interactions with the IgE Ce2 domain could be involved in restricting such conformational flexibility and influence the rates of binding and dissociation from the receptor. The range of motions and conformational arrangements of the Ce3 domains observed in these multiple crystal forms establish a set 20 of preferred arrangements which restrict possible models and approaches to blocking IgE binding to its receptor and to stimulating dissociation from the bound state.

While the various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur 30 to those skilled in the art. It is to be expressly understood, however, that such modifications are adaptations are within the scope of the present invention, as set forth in the following claims.

What is claimed is:

1. An isolated mutant IgE protein, wherein mutant IgE_{HC} proteins of said mutant IgE protein have reduced spatial mobility compared to the spatial mobility of unmodified IgE_{HC} proteins in an unmodified IgE protein, wherein said unmodified IgE_{HC}'s comprise the amino acid sequence of SEQ ID NO:11.
- 5 2. The isolated mutant IgE protein of Claim 1, wherein said mutant IgE protein is constrained to the open conformation or the closed conformation.
- 10 3. The isolated mutant IgE protein of Claim 1, wherein the N-terminal amino-acids residues of the Ce3 domains of said mutant IgE_{HC} proteins are unable to obtain an inter-residue distance of 23Å or more.
- 15 4. The isolated mutant IgE protein of Claim 1, wherein the N-terminal amino-acids residues of the Ce3 domains of said mutant IgE_{HC} proteins have a fixed, inter-residue distance of between about 13 Å and less than 23 Å.
- 20 5. The isolated mutant IgE protein of Claim 1, wherein said mutant IgE protein is constrained in a conformation in which the N-terminal amino-acids residues of the Ce3 domains of said IgE_{HC} proteins have an inter-residue distance selected from the group consisting of a distance of about 13Å, a distance of about 14Å, a distance of about 15Å, a distance of about 16Å, a distance of about 17Å, a distance of about 18Å, a distance of about 19Å, a distance of about 20Å, a distance of about 21Å a distance of about 22Å or a distance of between about 22 Å and less than 23Å.
- 25 6. The isolated mutant IgE protein of Claim 1, wherein said mutant IgE protein comprises a IgE_{HC} protein that comprises an amino acid sequence at least about 80% identical to SEQ ID NO:11, wherein the amino acid in said protein corresponding to position 2, 3, 4, 5, 6, 7, 8 or 9 or SEQ ID NO:11 is a cysteine or methionine.
- 30 7. The isolated mutant IgE protein of Claim 6, wherein said mutant IgE protein binds to an antibody raised against an IgE protein comprising an unmodified IgE_{HC} comprising the amino acid sequence of SEQ ID NO:11.
8. The isolated mutant IgE protein of Claim 1, wherein said mutant IgE_{HC} proteins comprise an amino acid sequence selected from the group consisting of SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:27.
9. An isolated nucleic acid molecule comprising a nucleic acid sequence at least about 80% identical to SEQ ID NO:10, wherein the codon in said nucleic acid

sequence corresponding to nucleotides 4-6, 7-9, 10-12, 13-15, 16-18, 19-21, 22-24, or 25-27 of SEQ ID NO:10 encodes a cysteine or a methionine.

10. The isolated nucleic acid molecule of Claim 9, wherein said nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of:

- 5 (a) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 4-6 of said nucleic acid sequence encodes a cysteine or a methionine;
- 10 (b) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 7-9 of said nucleic acid sequence encodes a cysteine or a methionine;
- 15 (c) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 10-12 of said nucleic acid sequence encodes a cysteine or a methionine;
- 20 (d) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 13-15 of said nucleic acid sequence encodes a cysteine or a methionine;
- 25 (e) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 16-18 of said nucleic acid sequence encodes a cysteine or a methionine;
- 30 (f) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 19-21 of said nucleic acid sequence encodes a cysteine or a methionine;
- 35 (g) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 22-24 of said nucleic acid sequence encodes a cysteine or a methionine; and
- 40 (h) a nucleic acid sequence at least about 90% identical to SEQ ID NO:10, wherein the codon at nucleotides 25-27 of said nucleic acid sequence encodes a cysteine or a methionine.

11. The isolated nucleic acid molecule of Claim 9, wherein said nucleic acid sequence encodes a protein having an amino acid sequence at least about 80% identical to SEQ ID NO:11, wherein the amino acid in said protein corresponding to position 2, 3, 4, 5, 6, 7, 8 or 9 of SEQ ID NO:11 is a cysteine or methionine and wherein said protein

binds an antibody raised against a protein having the amino acid sequence of SEQ ID NO:11.

12. The isolated nucleic acid molecule of Claim 9, wherein said nucleic acid sequence is selected from the group consisting of SEQ ID NO:12, SEQ ID NO:14, SEQ 5 ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24 and SEQ ID NO:26.

13. An isolated protein having an amino acid sequence at least about 80% identical to SEQ ID NO:11, wherein the amino acid in said protein corresponding to position 2, 3, 4, 5, 6, 7, 8 or 9 of SEQ ID NO:11 is a cysteine or methionine and wherein 10 said protein binds to an antibody raised against a protein having the amino acid sequence of SEQ ID NO:11.

14. The isolated protein of Claim 13, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) an amino acid sequence at least about 90% identical to SEQ ID 15 NO:11, wherein the amino acid at position 2 of such amino acid sequence is a cysteine or a methionine;

(b) an amino acid sequence at least about 90% identical to SEQ ID NO:11, wherein the amino acid at position 3 of such amino acid sequence is a cysteine or a methionine;

(c) an amino acid sequence at least about 90% identical to SEQ ID 20 NO:11, wherein the amino acid at position 4 of such amino acid sequence is a cysteine or a methionine;

(d) an amino acid sequence at least about 90% identical to SEQ ID 25 NO:11, wherein the amino acid at position 5 of such amino acid sequence is a cysteine or a methionine;

(e) an amino acid sequence at least about 90% identical to SEQ ID NO:11, wherein the amino acid at position 6 of such amino acid sequence is a cysteine or a methionine;

(f) an amino acid sequence at least about 90% identical to SEQ ID 30 NO:11, wherein the amino acid at position 7 of such amino acid sequence is a cysteine or a methionine;

(g) an amino acid sequence at least about 90% identical to SEQ ID NO:11, wherein the amino acid at position 8 of such amino acid sequence is a cysteine or a methionine; and

(h) an amino acid sequence at least about 90% identical to SEQ ID NO:11, wherein the amino acid at position 9 of such amino acid sequence is a cysteine or a methionine.

15. The isolated protein of Claim 13, wherein said protein comprises an amino acid sequence selected from SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25 and SEQ ID NO:17.

10 16. A method selected from the group consisting of:

(a) a method to identify a compound that inhibits the binding of IgE to a Fc ϵ RI, said method comprising:

(i) contacting an isolated mutant IgE protein of Claim 1 or an isolated protein of Claim 13 with a putative inhibitory compound in the presence of a Fc ϵ RI or Fc ϵ RI α protein; and

(ii) determining if said putative inhibitory compound inhibits the binding of said mutant IgE protein of Claim 1 or said isolated protein of Claim 13 to said Fc ϵ RI or Fc ϵ RI α protein; and

(b) a method to identify a compound that binds to IgE either in or resulting in a closed conformation, said method comprising:

(i) contacting an isolated mutant IgE protein of Claim 1 or an isolated protein of Claim 13 with a putative inhibitory compound in the presence of a Fc ϵ RI or Fc ϵ RI α protein; and

(ii) determining if said putative inhibitory compound binds to said mutant IgE protein of Claim 1 or said isolated protein of Claim 13.

25 17. The method of Claim 16, wherein said mutated IgE molecule comprises IgE_{HC}'s comprising an amino acid sequence at least about 90% identical to SEQ ID NO:11, wherein the amino acid in said protein corresponding to position 2,3 ,4, 5, 6, 7, 8 or 9 or SEQ ID NO:11 is a cysteine or methionine and wherein said protein binds an antibody raised against a protein having the amino acid sequence of SEQ ID NO:11.

30 18. An isolated compound that inhibits the binding of IgE to an Fc ϵ RI, wherein said compound is identified by the method of Claim 16.

19. The isolated compound of Claim 18, wherein said compound does not bind the open form of IgE.
20. A composition comprising the compound of Claim 18 and an excipient.
21. A method to protect an animal from a disease mediated by IgE, said
5 method comprising administering a composition of Claim 20.
22. A kit comprising an isolated mutant IgE protein of Claim 1 or an isolated protein of Claim 13 and a means to determine if a compound binds to said isolated mutant IgE protein of Claim 1 or said isolated protein of Claim 13.

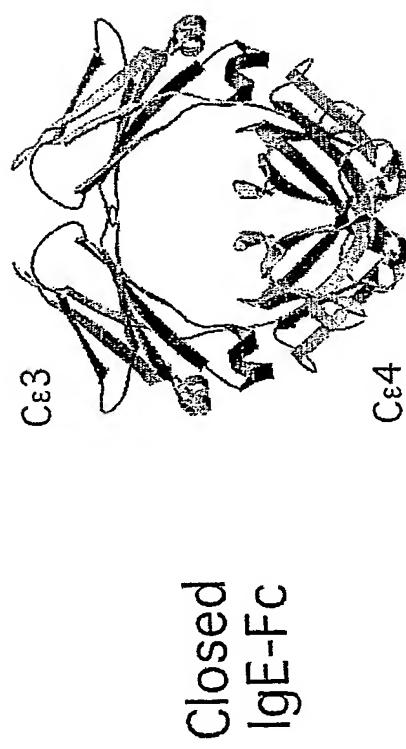


Fig. 1a

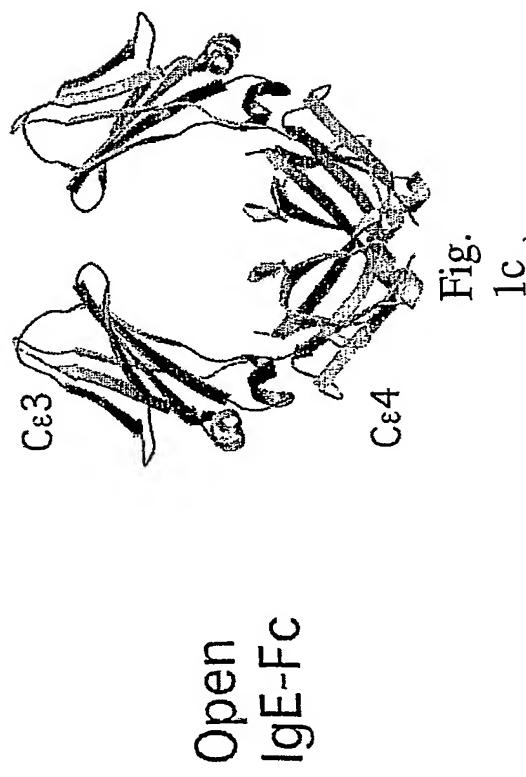


Fig. 1c.

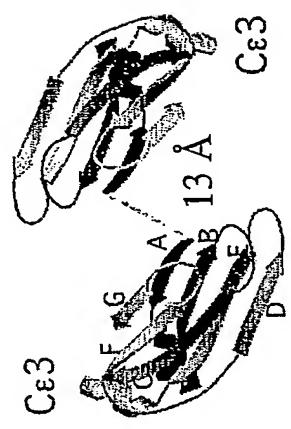


Fig. 1b

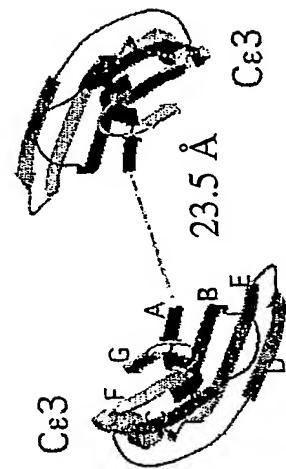
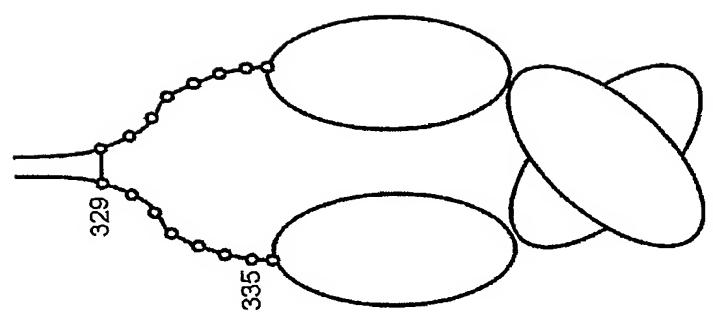
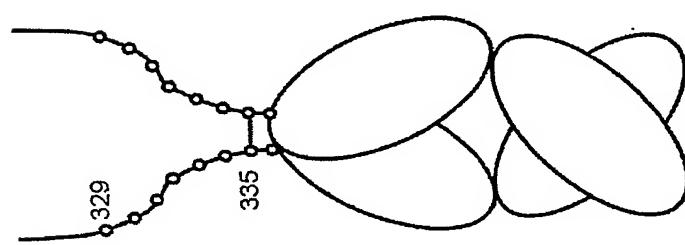


Fig. 1d



Cys 329
Open
Conformation

Fig. 2b



Cys 335
Closed
Conformation

Fig. 2a

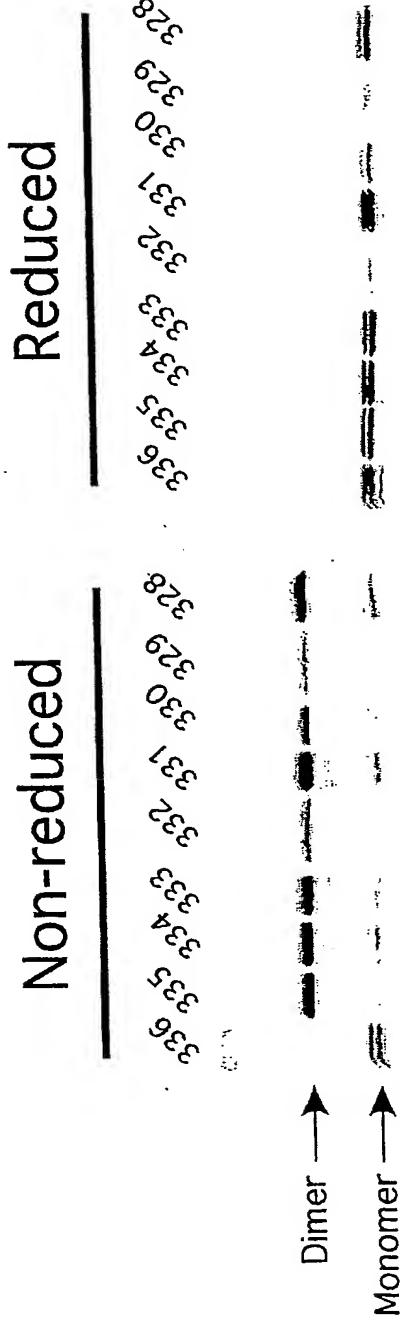


Fig. 3b

Fig. 3a

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